Recent insight into strategies for the design of antimicrobial peptides (AMPs)

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Abstract: With the increasing development of antibiotic resistance among key bacterial pathogens, there is an urgent need to discover novel classes of antibiotics. Although antimicrobial peptides (AMP) with their specific mode of action are considered major candidates for next-generation antibiotics, several challenges limit the use of these peptides for therapeutic applications.
In a large body of research, the focus is given to different approaches to the chemical modification of AMPs and how these modifications may improve the stability, antibiotic activity, proteolytic activity and prevent the cytotoxicity and side effects of AMPs. On the other hand, another group of research investigates the delivery of AMPs via nanocarrier systems as strategies used to enhance stability, control the release of peptides and reduce adverse peptide-related side effects, as well as improve their anti-microbial activities.
In the present article, we surveyed most recently published researches that provide us with good knowledge on structural features, mechanism of action, therapeutic aim, advantages and limitations, chemical modification approaches and carrying strategies of AMPs. Finally, according to Quality by Design, the most important potential effective factor and potential risk were mentioned in the development of AMP delivery systems.

Keywords: Antibiotic resistance, Antimicrobial peptides, Post-translational modification, Nanocarrier system, Quality by design

1 Introduction

Antibiotics are substances that treat infections by affecting bacteria through two main mechanisms: a bactericidal or a bacteriostatic one. Bactericidal antibiotics kill bacteria directly, while bacteriostatic antibiotics prevent their growth by inducing them into a stationary phase of growth [1,2]. *In vivo* as well as *in vitro* effectiveness, lack of toxicity and reasonable cost are vital features that antimicrobial agents must possess to provide an effective therapy [3]. The major concern about antibiotics is the ability of bacteria to develop resistance to them. This became one of the greatest challenges in the global health sector [4–6]. In the late 1960s and early 1970s, the significant success of antimicrobial drugs created a misleading belief that infectious diseases had been defeated. However, in the 2010s infectious diseases remained the second leading cause of death globally. Moreover, the emergence of antibiotic multi-resistance is increasing in different parts of the globe, thus creating a major concern as there are few or no treatments available for infections with certain microorganisms [7–9]. There are many factors that affect bacterial insensitivity to an antibiotic, including the spread of resistance genes and the over-prescription, overconsumption or misuse of antibiotics [10–12].

Since the global public healthcare system has been threatened by antibiotic-resistant bacteria, numerous researchers proposed to obtain new alternative agents which are called antimicrobial peptides (AMPs) and display a low rate of resistance development [13–15].

2 Antimicrobial peptides

AMPs can be categorized based on their source, target, structure, mechanism of action, therapeutic aim, modification and formulation methods. A schematic representation of different classifications of AMPs is shown in *Figure 1.* According to our present knowledge, this is a new overview of various AMP classifications.

2.1 Definition, history, source, target and mechanism of action

The relatively small size of antimicrobial peptides (<10kDa) made their isolation possible only in the 1980s. This was initially achieved in frogs, insects and granules of human and rabbit granulocytes. Since then, a large number of additional antimicrobial peptides has been found virtually everywhere in nature, amounting to over 2500 peptides known at present [16,17]. Antimicrobial peptides seem to have effector functions in innate immunity and can upregulate the expression of multiple genes in eukaryotic cells [18]. They represent a wide range of short, cationic or anionic, geneencoded peptide antibiotics. Despite sharing a few common features (such as cationicity, amphipathicity and short size), AMP sequences vary greatly, and at least four structural groups have been proposed to incorporate the diversity of the observed AMP conformation. As shown in *Figure 1*, AMPs are produced by bacteria and eukaryotes, such as protozoa, fungi, plants, insects and several types of vertebrate and invertebrate animals. They show a variety of targets, including Gram-positive and Gram-negative bacteria, parasites, fungi and some viruses [19–23]. AMP genes are present in the genetic material of a number of mammals. The expression of these genes has been detected in different cells, including neutrophils, monocytes, macrophages, epithelial cells, keratinocytes and mast cells. AMPs are synthesized as pre-pro-peptides and a post-translational process allows their final maturation into active peptides [24,25].

An insight into the mechanism of action of AMPs is essential for the further development and design of optimized AMPs that could be efficiently used as therapeutic drugs. Thus a broad range of researches are assigned to study the mechanism of action of AMPs [26–28]. According to these researches, AMPs are divided into two main groups based on their mode of action: membrane disruptive AMPs and non-membrane disruptive AMPs [29].

2.2 Structural and physicochemical features

The antimicrobial activity and selective toxicity of AMPs are significantly influenced by their structural and physicochemical features. Furthermore, studying different structural parameters of AMPs is a vital part of the design and development of novel antimicrobial agents with enhanced antimicrobial activity [30].

2.2.1 Conformation (X)

Based on secondary structures assumed by AMPs in the presence of other biological membranes, they are categorized into different conformations, such as α-helix, β-sheet, extended helix and loop (*Figure 1 and 2*) [25–27].

Circular Dichroism (CD), X-ray crystallography and Nuclear Magnetic Resonance (NMR) studies are commonly used to determine the secondary structure of these peptides [30,31] The α-helical AMPs, including cecropin and pexiganan, tend to form amphipathic helices in certain organic solvents, such as trifluoroethanol. These α-helical AMPs disturb the bacterial membrane by employing various mechanisms of action, including the formation of barrel-like bundles (barrel-stave model), carpet-like clusters (carpet model) and toroidal pores (toroidal pore model) into the membrane.

AMPs with **β-sheet structure**, such as α-, β-defensins and protegrin, form β-hairpin structures stabilized by disulfide bridges. Most of the β-sheet AMPs have a rigid structure and the bacterial membrane is disturbed by a perpendicular insertion into the lipid bilayer and the formation of toroidal pores.

Mixed structure (αβ-peptides) AMPs, such as bactenecin, adopt a loop formation with one disulfide bridge [32,33].

The **extended AMPs**, which are rich in specific amino acids, have irregular secondary structures. Many of these peptides show antimicrobial activity only after interacting with the membrane and undergoing consequent conformational changes. Indolicidin with 13 amino acids, a member of this group of AMPs, contains five tryptophan and three proline residues. The peptide adopts a poly-L-II helical structure in the presence of liposomes, and the high content of tryptophan residues is responsible for their interaction with lipid membranes.

2.2.2 Charge

Many of the antimicrobial peptides display a net positive charge, ranging from 2 to 9, and may contain highly defined cationic domains. Cationicity is essential for the initial electrostatic attraction of antimicrobial peptides to negatively charged phospholipid membranes of bacteria and other microorganisms [30,34–38]. However, this relationship is not fully linear. Within a certain range,

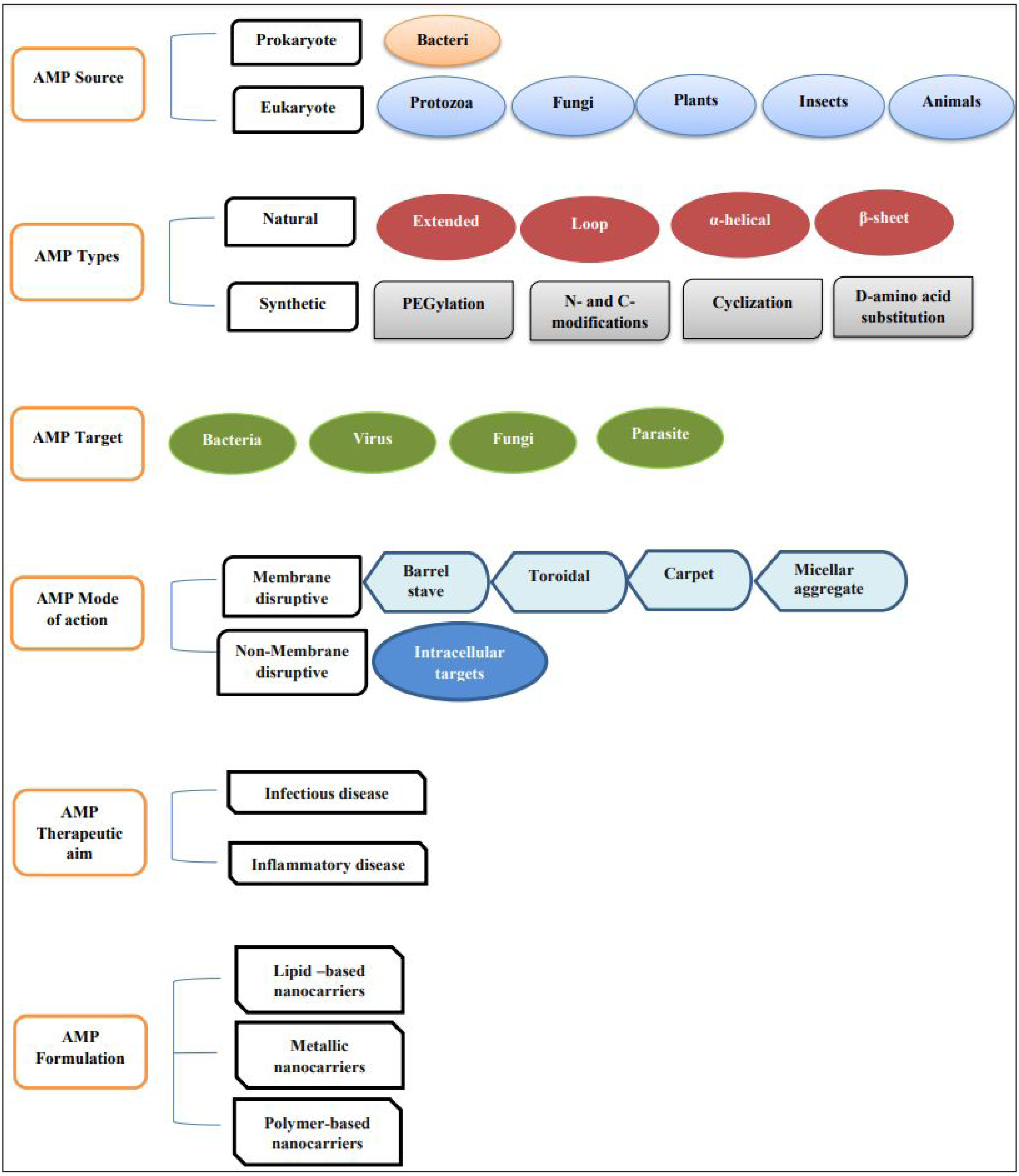

Figure 1 Schematic representation of different classifications of antimicrobial peptides. AMPs can be categorized based on their source, target, structure, mechanism of action, therapeutic aim, modification and formulation type

increasing peptide cationicity is generally associated with increasing antimicrobial strength. For instance, studies with magainin 2 analogs show that increasing the charge from 3 to 5 results in increasing antibacterial activities against Gram-negative and Gram-positive pathogens. However, a net charge from 6 to 7 leads to an increase in the hemolytic propensity and to a loss of antimicrobial activity. Therefore, it can be concluded that there is a risk beyond which increasing the positive charge no longer increased the activity of AMP [32]. Although in a wide majority of cases

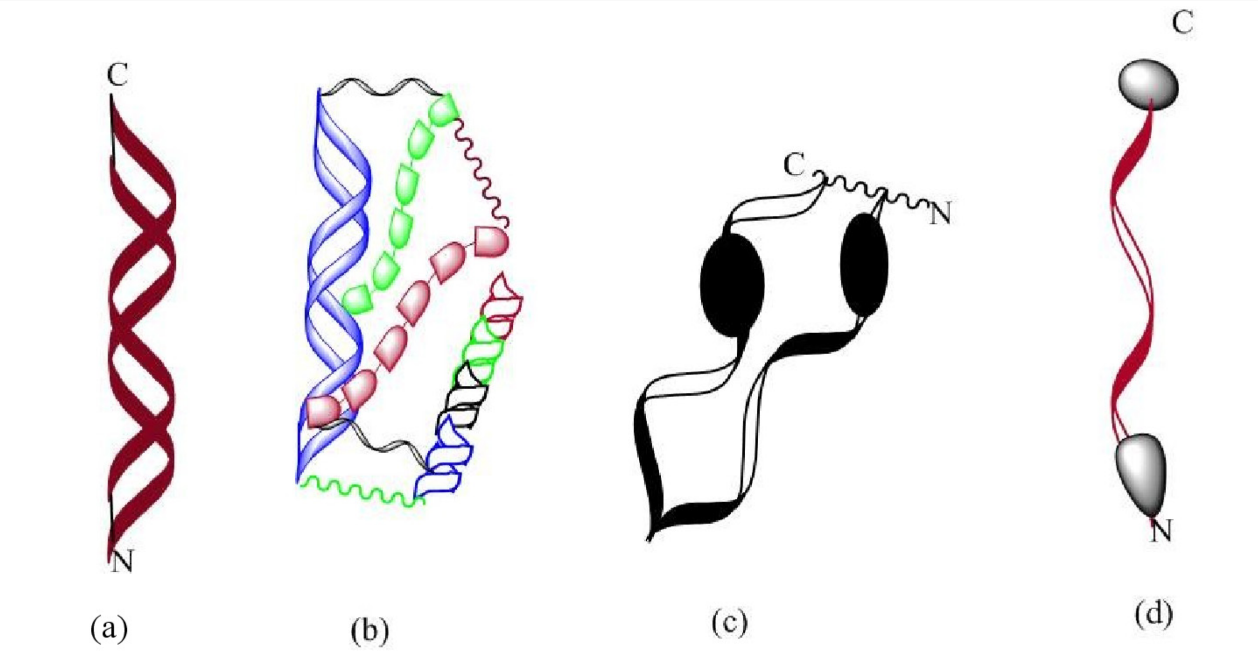

Figure 2 The four structure classes of AMPs. (a) α-helical (LL-37), (b) β-sheet or loop or turn (Protegrin-1), (c) αβ-peptides (mixed-structure) (Microcin J25) and (d) extended (non-αβ-peptides) (Indolicidin)

AMPs are cationic, anionic AMPs (AAMPs) have also been described as an integral and important part of the innate immune system and increasingly identified in vertebrates, invertebrates and plants over the last decade [39]. While cationic peptides are rich in arginine and lysine, AAMPs are small peptides rich in glutamic and aspartic acids. AAMPs that are complexed with zinc, or highly cationic peptides, are often more active than neutral peptides or those with a lower charge [40].

2.2.3 Amphipathicity (A) and hydrophobicity (H)

In a research by Mihajlovic *et al.*, the amphipathicity of antimicrobial peptides plays a crucial role in pore formation and can also contribute to a better understanding of the mode of action in antimicrobial peptides [41,42]. Kondejewski *et al.* have reported that the antimicrobial activity and toxicity of peptides are notably enhanced with amphipathicity. However, an extremely amphipathic nature is not desirable in cyclic AMPs since it decreased the specificity and increased interactions with outer membrane components [43].

Hydrophobicity is a main feature for the effective membrane permeabilization of AMPs as it determines the extent to which a peptide can partition into the lipid bilayer. However, an increase in the levels of hydrophobicity is strongly related to mammalian cell toxicity and loss of antimicrobial specificity. Therefore, moderate hydrophobicity is needed against the bacterial membrane [32]. A research by Wood *et al.* on a linear cysteine-deleted tachyplesin (CDT), examined the effect of hydrophobicity on antimicrobial activity. Analogs with hydrophobic isoleucine residues placed throughout the sequence of CDT showed comparable antimicrobial activity to CDT but lower hemolysis [44].

AMPs with moderate features (charge, hydrophobicity, amphipathicity) and a good balance between these characteristics showed higher antimicrobial activity and lower cytotoxicity and hemolysis in mammalian cells [36,45].

2.3 Therapeutic aim

As shown in *Figure 1* and *Table I*, AMPs can be categorized based on their therapeutic aim. Recent researches have demonstrated that, in addition to the antimicrobial functions of AMPs, these peptides also play an important role in the complex pathogenesis of several inflammatory diseases [46,47]. According to the results of a research project, the sustained release of drugs at the site of action presented excellent results in the treatment of chronic wounds [48]. In the table below, the association between some of the most common AMPs with different conditions, including infectious and inflammatory diseases, is listed and classified

Table I *Some of the most recent researches showing various AMPs and their administration routes effective in the treatment of acute or chronic diseases*

Peptide	Disease	Chronic/Acute	Administration Route	Ref.
Rhesus theta defensin-1 (RTD-1)	Acute lung injury (ALI)	Acute	Parenteral	[51].
Cathelicidin LL-37	Acute thrombosis			[52]
Catestatin (CST)	Acute and chronic pain			[53]
Human beta defensins 1 (HBD1)	Acute HIV-1 infection			[54]
Human cathelicidin (hCAP18/LL-37)	Chronic obstructive pulmonary disease (COPD)	Chronic	Inhalation	[49]
HBD1, HBD2, HBD5 and HBD6	Crohn's disease (CD)		Oral	[50]
hBD-3	Wound		Dermal	[55]
β defensin	Chronic rhinosinusitis (CRS)		Nasal	[56]

into two groups of acute and chronic diseases. Based on literature review, AMPs which are used to treat chronic diseases should provide controlled and sustained release by choosing the proper administration route, while an immediate release formulation of AMPs is effective for acute diseases. Thus the therapeutic aim of AMPs has significant influence on formulation parameters in the delivery of these peptides, and depending on the medical application of AMPs, the drug release profile and therefore the administration route are different [49,50].

3 Advantages and limitations of AMPs

In relation to small molecule drugs, peptide therapeutics has considerable advantages in terms of safety aspects. Since the products resulting from their degradation are natural amino acids with a short half-life, only a small quantity of peptides is accumulated in the tissues. The result is a reduction in the safety risks caused by metabolites. Less immunogenicity is another advantage of therapeutic peptides. Generally, even synthetic peptides are less immunogenic than recombinant proteins and antibodies. Among different peptides, AMPs emerged as essential tools with a broad-spectrum of activity and a low rate of resistance development [34,57]. Besides the mentioned advantages, AMPs have limitations, such as low metabolic stability and low permeability across biological barriers, high costs and poor relevance of antimicrobial activity of AMPs *in vivo* and *in vitro*, cytotoxicity and difficulty in reaching targeted sites at active concentration due to degradation. In the next chapter, it is shown that these initial barriers are being increasingly overcome with new chemical modification strategies for the development of stable, more cost-effective and potent broad-spectrum synthetic peptides [58]. The following figure shows the critical points to consider during formulation to obtain a good quality product (Figure 3).

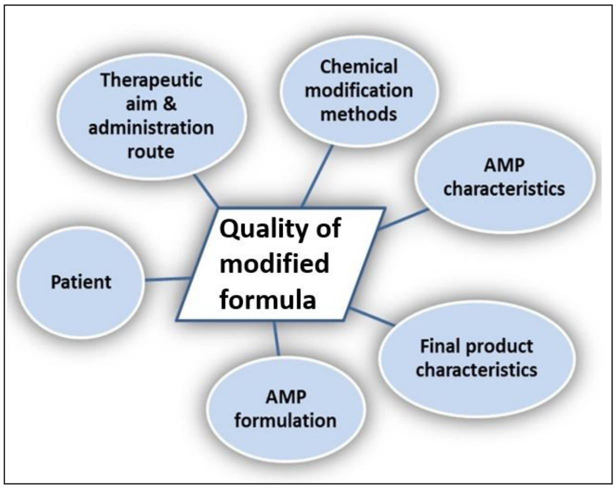

Figure 3 Critical points to consider during formulation

4 Post-translational /chemical modifications of AMPs

Strategies such as N- and C-modifications, incorporation of non-natural or D-amino acids, cyclization and attachment of the polyethylene glycol polymer to peptides (PEGylation) allowed several researchers to enhance the bioavailability of AMPs and improve both their bio-distribution and rate of clearance. Polyethylene glycol (PEG) protects peptides from serum proteases and releases them in a traceless fashion with full bioactivity. PEGylated peptides display a longer circulation time, in which water solubility, stability, resistance, biocompatibility, minimal toxicity and immunogenicity of the peptide are improved [59]. The proteolytic degradation of peptides can be decreased by protecting their C- and N-terminus with acetylation or amidation. Also, modifying

Table II *Some of the most recent researches showing various AMPs, their target, their administration route, different carrier systems for loading them and their advantages, risks and perspectives of further development*

Peptide name	Target /disease	Nanocarrier system	Advantages	Risks and future perspective	Ref.
Esculentin-1a, Esc(1-21)	*P. aeruginosa* (treatment of epithelial infections and healing of the injured tissue)	Soluble AuNPs covalently conjugated to AMP ***via*** a poly(ethylene glycol) linker	- Highly enhanced antipseudomonal activity - Preserved mode of action of the free peptide, without being toxic to human cells - Accelerated recovery of an injured skin layer - Resistant to proteolytic digestion - Biocidal against a wide range of microbial pathogens - Ease of AuNPs synthesis - Biocompatibility	- Possibility of inefficient delivery of AMPs to the target infectious site - Lack of information on the basic rules governing molecular interactions between such coated-AuNPs and cells or complex tissues	[82]
Ubiquicidin 29–41 (UBI)	*E. coli*, *P. aeruginosa*	Engineered multivalent silver nanoparticles functionalized with UBI	- Enhanced antimicrobial activity - High affinity and selectivity towards bacterial infection - Synergic effects against microorganisms	- Microbicidal effects are limited to certain types of microorganisms - Lack of understanding of the structure of multivalent nanoparticles and stabilization mechanisms	[83]
LL-37 (LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLVPRTES	*E. coli*, *S. aureus*	Peptide-loaded mesoporous silica nanoparticle	- Antimicrobial effects can be controlled in peptide-loaded mesoporous silica nanoparticle systems	- In the case of positive charge, mesoporous silica nanoparticles can cause toxicity against the human erythrocytes	[85]
LL-37 (LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLVPRTES	*E. coli*, *S. aureus*	Mesoporous silica containing LL-37	- Potential as an implantable material or surface coating - Controlling implant-related infections, e.g., for multiresistant *S. aureus*	- Low toxicity	[86]
HHC-36 (KRWWKWWRR)	*S. aureus*	HHC-36 loaded self-organized, vertically oriented titanium TiO_2 nanotube	- Slow release profile from 4 hours up to 7 days - It can be applied on the surface of implants as locally delivered antimicrobial agent for peri-implant infections	-	[87]
Indolicidin		Carbon nanotube-indolicidin conjugate	- It can improve the efficacy of indolicidin at 1000-fold less concentration than the free indolicidin	- In the future it will be tested in animal model	[88]
TP359, TP226 and TP557	*S. aureus*	AMP-functionalized silver-coated carbon nanotubes	- Non-toxic - Help reduce the infection on the skin model	- Further evaluating the antibacterial potential of AMP-functionalized silver-coated carbon nanotubes in a time-dependent manner (longer incubation times)	[89]

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Peptide name	Target /disease	Nanocarrier system	Advantages	Risks and future perspective	Ref.
Cationic peptides	*S. aureus*	Fullero-peptide containing cationic AMP with solid-phase synthesis	- Fullero-peptides could be easily purified and tested for their biological activity	- The resins have shown a strong tendency to retain the fullerene-based peptides, it can be a problem during the removal step	[90]
Leucine-rich antimicrobial peptide (FALALKALKKALKKLKKALKKAL)	*E. coli, S. aureus, MRSA*	Paramagnetic nanoparticles encapsulated in cationic liposomes	- Enhanced hemocompatibility and antimicrobial activity - Liposomal envelope protects the cargo against unwanted interactions with the environment resulting in the prolonged persistence of the cargo in the body	- Toxicity risks and adverse effect of advanced nanomaterials on the therapeutic index of peptides	[93]
Nisin Z	*S. aureus, S. epidermidis*	Nano-structured lipid carriers (NLCs)	- Enhanced stability, solubility, antimicrobial activity, biodegradability and selectivity of peptide towards bacterial cells - Synergism was observed for the combination of nisin Z with conventional antibiotics - No toxicity in mammalian cells	- No activity against Gram-negative bacteria. However, the activity towards Gram-negative bacteria can be enhanced by using the chelating agent ethylenediaminetetraacetic acid (EDTA). Therefore more studies are required on incorporating nisin Z and EDTA in NLCs simultaneously and testing the effectiveness *ex vivo* and *in vitro* for topical application	[94]
Human cathelicidin LL-37	Infection, immunity and wound repair	pH-tunable nanocarriers named nano-biointerfaces (OA/LL-37 self-assemblies)	- Enhanced solubility and antimicrobial activity - Protection of the peptide from degradation by partitioning into the hydrophobic or the hydrophilic sections of the self-assemblies, or by localization at their water-lipid interfaces - Directing the antimicrobial activity to the affected tissues, while minimizing toxicity	- Limitations in controlling and triggering self-assembly	[95]
GIBIM-P5S9K	*E. coli, MRSA, P. aeruginosa* (infectious diseases caused by resistant microorganism)	PLA and PLGA NPs	- Enhanced antimicrobial activity - Protection of peptide against degradation - Slow release - Biodegradability - Biocompatibility - No toxicity - Hemocompatibility	- Limitations of natural polymer NPs, such as risk of purity variation - Further modification of PLGA-NPs can control the structure, encapsulation degree, administration route, drug release and degradation rate	[97]
S16 and S32	*ESKAPE* group *MDR* and *CMDR*	Star-shaped peptide polymer nanoparticles synthesized via ring-opening polymerization	- Enhanced antimicrobial activity - No resistance acquisition by CMDR bacteria - Low toxicity - Low-cost - Selectivity of the peptides towards pathogens over mammalian cells - Applications in nanomedicine, particularly in the fields of gene therapy	- Lack of information on the exact mechanism of membrane disruption	[98]

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Peptide name	Target /disease	Nanocarrier system	Advantages	Risks and future perspective	Ref.
Colistin	*P. aeruginosa*	PLGA nano-embedded microparticles containing colistin	- It displays prolonged efficacy in biofilm eradication compared to the free colistin	- It can be a novel antimicrobial formulation for *P. aeruginosa* lung infection in cystic fibrosis patients	[99]
Nisin	*E. coli, Listeriamonocytogenes*	Nisin/g-PLG nanoparticle	- The release of nisin from the nanoparticles was pH-dependent	- It could be a promising food preservative	[100]
APO (All peptides optimized, name of designer antimicrobial peptide), colistin	*Acinetobacter baumannii*	APO monomer-impregnated nanofiber dressing	- It resulted in significantly reduced wound size and wound bacterial load	- It can be developed as an economical first-line treatment option to skin injuries in general	[101]
ε-poly(L-lysine) (ε-PL)	*S. aureus, S. epidermidis, E. coli*	ε-PL functionalized poly(acrylic acid) /poly(vinyl alcohol electrospun nanofibers	- It did not display cytotoxicity to human corneal epithelial cells - EC10 and EC50 in the order *S. epidermidis* > *S. aureus* > *E. coli*	- In the future it can be used in biocompatible nanofibrous dressings with durable antibacterial and antifouling efficiency, and potential application as wound dressings or other medical uses	[102]
Novicidin	*E. coli, S. aureus*	Octenyl succinic anhydride-modified hyaluronic acid nanogels loaded novicidin	- It is colloidally stable in a physiological ionic strength buffer - It displays sustained release over 12 days - Reduced cytotoxic effects - Relatively high drug load	- In the future other amphipathic AMPs may also be successfully applied with this method	[104]
LLKKK18	*M. avium, M. tuberculosis*	Self-assembling hyaluronic acid nanogels loaded LLKKK18	- Intratracheal administration of nanogel significantly reduced infection levels in mice after just 5 or 10 every other day administrations	- It may hold great potential as an alternative approach to control tuberculosis and other mycobacterioses	[105]
Poly(Lys-Ala) polypeptides	*E. coli, S. aureus* (wound healing)	Cell-adhesive hydrogel (formed by cross-linking of poly(Lys)$_{60}$-(Ala)$_{40}$ and 6-arm PEG-ASG)	- Enhanced cell adhesion and proliferation accelerating wound healing - Enhanced antimicrobial activity - Ease of the hydrogel synthesis - Inherent antibacterial activity of the hydrogel - Low cost	- Lack of kinetic studies on release profile - Further studies are in progress to enhance cell adhesion through the incorporation of additional biological moieties	[106]
Tet213	*S. aureus*	RADA16-AMP self-assembling peptide hydrogel	- The synthetic process was simple without the limitation of time or temperature - It could be used conveniently and easily for patients	- It could be used as a promising material for bone infection and osteomyelitis treatment	[108]

the sequences of peptides by the substitution of natural L-amino acids for their D enantiomers, α/β-substituted α-amino acids or even β-amino acids are other similar approaches that result in overcoming peptide hydrolysis. D-amino acid substitution in a peptide may influence not only the stability of the peptide but also its secondary structure and therefore its ability to incorporate into membranes [60–63]. It is worth mentioning that in addition to the modification strategies described in this review article, the use of other types of AMP modification, such as computer-assisted methods, has been increasing significantly[64].

4.1 N- and C-modifications

In a new finding by Kuzmin *et al.*, N-terminal acetylation and C-terminal amidation significantly increased the stability and hemolytic activity of the modified AMP in human serum. In addition, the hemolytic activity and specific and non-specific cytotoxicity of the peptide increased [65]. In another research, different series of branched tetramers of a proline-rich antimicrobial peptide (PrAMP) named Chex1-Arg20 was studied against a number of Gram-negative nosocomial pathogens. C-terminal PrAMP hydrazidation together with its tetramerization resulted in both broad-spectrum antibacterial selectivity and potency of PrAMP action [66]. The relationship between AMP property and chemical modification indicates peptide engineering. Depending on the aim of our design, peptides could be modified with various methods so that the desired features can be enhanced whereas side effects can be reduced [67].

4.2 Cyclization

Cyclization of the linear peptide HAfp$^{1-23}$_KK resulted in a cyclic peptide with considerably improved antibacterial activity and minimum inhibitory concentration (MIC) value against multidrug-resistant *Pseudomonas aeruginosa* (*P. aeruginosa*) and methicillin-resistant *Staphylococcus aureus* (*S. aureus*). The designed cHAfp$^{1-23}$_KK also exhibited very low cytotoxicity with respect to its MIC values determined against different bacteria [68]. In another new study, C-MPI-1 and C-MPI-2, cyclic analogs of a natural AMP named Polybia-MPI (MPI), were synthesized by the click chemistry approach. As a result, C-MPI-1 showed improved stability against trypsin in comparison with the parent peptide. Moreover, MPI displayed sustained antimicrobial activity while C-MPI-2 displayed no antimicrobial activity [35]. Cyclization can show significant effects on enhancement of antibacterial activity, MIC value, stability and cytotoxicity of AMPs. However, there is a risk of losing the activity of the peptide after the cyclization of AMPs. With the application of cyclisation, Chan *et al.* could improve stability and enhance cell toxicity against a cancer cell line without toxicity on a noncancer cell line and they could improve the antimalarial effect of gomesin [69].

4.3 D-amino acid substitution

D-amino acid substitutions can result in antimicrobial peptides resistant to proteolysis [63,70]. A recent study shows that a proline-rich antimicrobial peptide, A3-APO (a discontinuous dimer of the peptide Chex1-Arg20), underwent degradation to small fragments at positions Pro6-Arg7 and Val19-Arg20. To minimize this degradation, a series of Chex1-Arg20 analogs were prepared via Fmoc/tBu solid phase peptide synthesis with D-arginine substitution at these sites. The activity of the peptides decreased against Klebsiella pneumonia by the replacement of arginine at position 7, while substitution at position 20 did not greatly affect the activity. Moreover, none of these peptides showed any cytotoxicity to mammalian cells. These findings can result in the development of more effective and stable peptide analogs with further substitution at position 20 [71]. Thus depending on the position of substitution, D-amino acid substitution can result in the sustained or decreased antibacterial activity of AMPs. Moreover, this type of modification is an effective means for decreasing the cytotoxicity of AMPs. Jia *et al.* applied D-amino acid substitution to improve the stability of polybia-CP. The results demonstrated that all D-amino acid derivatives and partial D-lysine substitution derivatives have improved stability against trypsin and chymotrypsin[72].

4.4 PEGylation

A proline-rich AMP named Bac7(1e35) (which protects mice against *Salmonella typhimurium* infection) was linked to a PEG molecule via a cleavable ester bond or via a non-hydrolysable amide bond. Both PEGylated derivatives exhibited the same mode of actions. However, the antimicrobial activity of the releasable conjugate is higher than that of the stably linked one. Moreover, both derivatives exhibited a lower clearance rate and wider distribution in mice compared to the unmodified peptide [73]. Although the PEGylation of peptide drugs prolongs their circulating lifetimes in plasma, it can mask the binding site in peptides and therefore extremely reduce the activity of the peptide (PEGylation of biopharmaceuticals). Thus, in order to circumvent the conflicting effects of PEGylation, different strategies such as changing the size and the location of the PEG molecule were offered in several researches. For instance, an antimicrobial synthesized peptide named MA was modified with low molar mass PEG chains. The PEGylated peptides self-assembled in aqueous solution into micelles with a PEG shell and a peptide core, resulting in the increased photolytic stability of the AMP due to the shielding protection of the core peptide by the PEG shell, leading to the increased antimicrobial activity and the decreased hemolytic activity of AMP [74]. In another recent research project, by using the PEG-Linker-Drug strategy, the linker sequence can be optimized for a given therapeutic peptide named Onc112 providing release rates from <1 h to >40 h [75]. It can be concluded that PEGylation significantly increases the half-life of AMPs, with potential improvement in bioavailability and distribution but without adversely affecting the binding and the activity of peptides. Factors such as the molecular weight of PEG molecules and the type of linkage for the attachment of PEG to AMPs improve the performance of PEGylated AMPs and overcome limitations such as masking the binding sites of peptides by PEG molecules. Thus the advantages of PEGylation outweigh its limitations and make it a promising method of modification. Most PEGylated proteins are designed with a covalent bond between the PEG molecule and the protein or peptide. In many cases the PEGylation method can cause decreased functional activity. Therefore the releasable PEGylation method was developed. In this case, the PEG molecule can be released over time in the circulation and the therapeutic protein can keep its activity [76]. Gong *et al.* prepared releasable PEGylated arginine-rich AMPs. These conjugates were insensitive to serum proteases and the AMP could be released with fully functional activity [77].

5 Strategies for AMP delivery

The diversity of novel formulations within the limits of nanotechnology may also provide novel applications going beyond antimicrobial activity [78,79]. In the following, some of these researches using different nanocarrier systems are discussed. More evaluations on the advantages and risks related to each approach are listed in *Table II*.

5.1. Inorganic nanoparticles

5.1.1 Metallic nanoparticles

AMP delivery strategies can be implemented with inorganic materials, one of which is gold nanoparticles (NPs) (Figure 4). Gold nanoparticles can be functionalized (Figure 5) by beta-amyloid peptides or pentapeptide fragments [80]. On the other hand, metallic NPs by themselves are known to have antimicrobial activities and thus disrupt the bacterial cell membrane and cause cell penetration or react with intracellular targets and cause toxicity. The immobilization of AMPs to metallic nanoparticles might therefore represent an alternative solution in the fight against antibiotic resistant pathogens and could also improve the antimicrobial activity of both components. Moreover, immobilization to nanoparticles could also help to overcome some limitations of AMPs, such as susceptibility to proteases

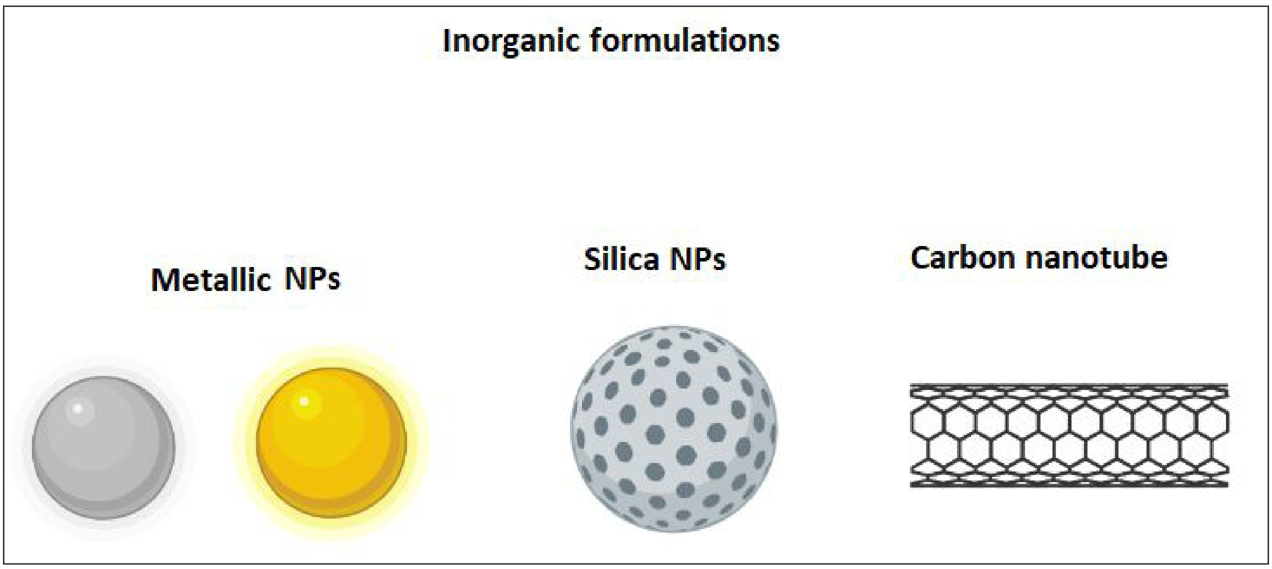

Figure 4 Inorganic formulations (Created with BioRender.com)

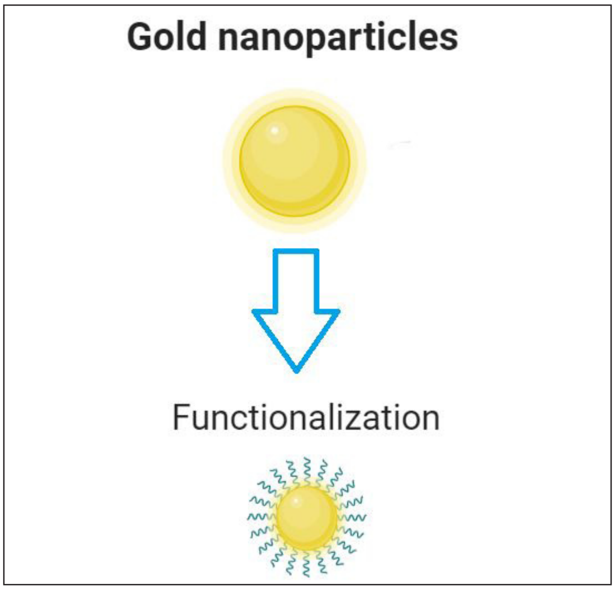

Figure 5 Functionalization of gold nanoparticle (Created with BioRender.com)

and poor permeability across biological barriers [81,82]. A derivative of the frog skin AMP esculentin-1a, Esc(1-21), covalently conjugated to soluble AuNPs *via* a poly(ethylene glycol) linker, results in a significant rise in the activity of AMP against the motile and sessile forms of *P. aeruginosa* without being toxic to human keratinocytes. Moreover, the peptide displayed more resistance to proteolytic digestion and disintegrated the bacterial membrane at a very low concentration. Wound healing activity on a keratinocyte monolayer is another advantage of engineered AuNPs [82]. In a recent research, an increase in antibacterial activity against Gram-negative bacteria appeared when silver nanoparticles were capped with cationic antimicrobial peptide ubiquicidin 29–41 (UBI). This probably results from the multimeric or polyvalent arrangement of ligands distributed on the metallic NP surface [83].

5.1.2 Silica nanoparticles

Silica NPs can be appropriate carriers of AMPs because they have well-defined mesopores in nm range, are chemically stable and relatively biocompatible [84]. Braun *et al.* found that the surface charge of mesoporous silica NPs strongly influence the loading of the LL-37 AMP into mesoporous silica NPs and the release onto the surface [85]. Izquierdo-Barba *et al.* developed a novel one-pot evaporation induced self-assembly method for the preparation of mesoporous silica reservoir, which can be applied for encapsulating antimicrobial agents and AMP (LL-37) [86].

5.1.3 TiO_2 nanotube, carbon nanotube

Ma *et al.* established that TiO_2 nanotubes can be used as potential carriers of AMPs. The AMP (HHC-36) was loaded into the TiO_2 nanotubes with vacuum-assisted physical adsorption. The AMP-loaded TiO_2 decreased the adhesion of *S. aureus* on the surface and could kill bacteria significantly [87]. Sur *et al.* prepared carbon nanotube-indolicidin and gold nanoparticles-indolicidin conjugates and these conjugates were found to be able to improve the efficacy of indolicidin at 1000-fold less concentration than the free indolicidin [88]. Chaudhary *et al.* prepared silver-coated carbon nanotube functionalized with AMP (TP359, TP226 and TP557). They investigated toxicity, morphology with scanning electron microscopy and antibacterial activity against *S. aureus*. The results showed that the silver coated carbon nanotube functionalized with antimicrobial peptides was non-toxic and helped reduce the infections [89]. Pantarotto *et al.* successfully performed the solid-phase synthesis of fullero-peptides containing cationic AMP. They have specific activity against Gram-positive bacteria and can therefore be anti-infective agents [90].

5.2 Organic materials

5.2.1 Lipid structures

MP delivery strategies can also be implemented using organic materials. One group of them is lipid systems (Figure 6). Liposomes reduce toxicity, extend drug half-life, possess biocompatibility and biodegradability [91]. They have also proved to be capable of improving the delivery of bioactive molecules by functioning as circulating micro-reservoirs for sustained release [92]. A delivery system with paramagnetic nanoparticles encapsulated in cationic liposomes tested with the leucine-rich antimicrobial peptide (FALALKALKKALKKLKKALKKAL) results in better hemocompatibility (7.5%) and antimicrobial activity of the entire complex against *Escherichia coli (E. coli), S. aureus* and methicillin-resistant *S. aureus* (MRSA) compared to conventional penicillin antibiotics. However, further in vivo experiments are required to specify the real effect of advanced nanomaterials on the therapeutic index of peptides [93] (*Table II*).

In another study, nanostructured lipid carriers (NLCs) were investigated as a delivery system for

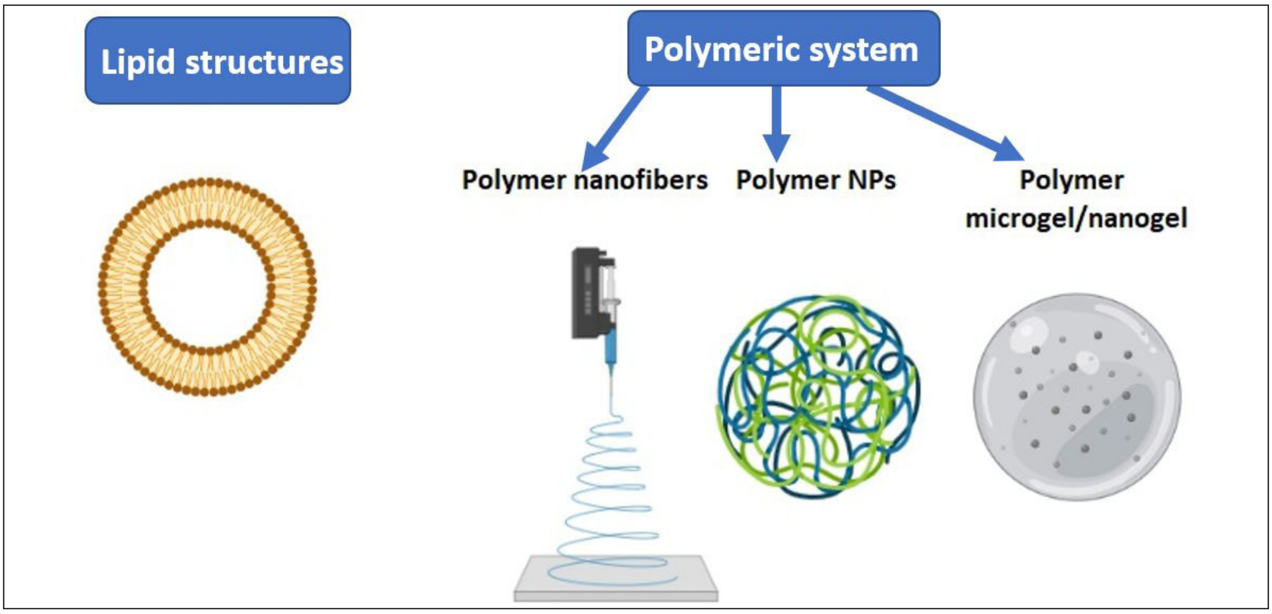

Figure 6 AMP formulations containing organic materials (Created with BioRender.com)

an antimicrobial peptide named nisin Z against two Gram-positive species found on the skin surface, *S. aureus* and *Staphylococcus epidermidis* (*S. epidermidis*) at physiological pH. The results proved the effectiveness of NLCs as promising biodegradable delivery systems for the enhancement of the stability, solubility and antimicrobial activity of AMP and also as promising potential systems for dermal applications [94].

In addition to liposomes, micelles also show great potential as vectors for drug delivery, especially for poorly water-soluble drugs because of their size and their ability to solubilize hydrophobic drugs and to achieve target or site-based drug delivery. For instance, the potential delivery of human cathelicidin LL-37, and its protection from degradation was investigated by Gontsarik *et al.* Nanocarriers named nano-biointerfaces were prepared through the self-assembly of oleic acid (OA) with human cathelicidin LL-37 in an excessive amount of water. According to the results, hydrophobic and electrostatic interactions between OA and the peptide molecules drove the detected structural transformations (from normal emulsions via micellar cubosomes and hexosomes to vesicles) with both composition and pH. These structural changes are interesting for the further development of pH-driven nanocarriers for the targeted delivery of poorly water-soluble AMPs as an alternative to conventional antibiotics. They may also be valuable for the further understanding of the mechanism behind the AMP-driven destruction of the bacterial membrane [95].

5.2.2 Polymeric systems

5.2.2.1 Polymeric nanoparticles

Polymeric NPs are often used as carriers for protein and peptides [96] that can be prepared by different techniques such as emulsion/solvent evaporation, double emulsion, solvent spread, coacervation, nanoprecipitation, ionotropic gelation and salt precipitation [97]. A new AMP named GIBIM-P5S9K was loaded into polylactic acid (PLA) and poly (lactic-co-glycolic) acid (PLGA) NPs via the double-emulsion solvent evaporation method resulting in a release of around 50% of the peptide from the NPs during the first 8 hours. These peptide-loaded NPs presented higher antibacterial activity than the free peptide against *E. coli*, MRSA. Other advantages listed in *Table II* suggested these synthesized NPs as a promising candidate for AMP delivery and a protection system against enzymatic peptide degradation [97]. A new class of antimicrobial agents, termed 'structurally nano-engineered antimicrobial peptide polymers' (SNAPPs), was synthesized in the form of 16- and 32-arm star peptide polymer nanoparticles (S16 and S32) and showed sub-μM activity against Gram-negative bacteria, including a group of pathogens responsible for the majority of hospital-acquired infections (referred to 'ESKAPE' pathogens) and colistin-resistant and multidrug-resistant (CMDR) pathogens, while demonstrating low toxicity [98]. Angelo *et al.* prepared PLGA nano-embedded microparticles containing cationic AMP (colistin) as a lung delivery system. It is a promising formula because it can help diffusion through the mucus and the bacterial biofilm. These particles can be appropriate for the treatment of lungs infected with *P. aeruginosa* in cystic fibrosis [99]. The aim of another research group was to improve the antimicrobial efficiency of nisin with encapsulation in a poly-g-glutamic acid (g-PGA) and chitosan nanoparticle using the self-assembly method. The dissolution of nisin from these formulations was pH-dependent. It was found that the g-PGA/chitosan nanoparticle containing nisin had higher antimicrobial efficiency than the g-PGA nanoparticle containing nisin [100].

5.2.2.2 Polymer nanofibers

Sebe *et al.* formulated polyvinyl alcohol nanofiber-loaded AMP (proline-rich peptide dimer A3 APO) and it was polymerized into a solid patch dressing. It was tested in wounds of mice infected with multidrug resistant *A. baumannii* and the results revealed that the patch containing APO improved the wound appearance significantly more than the patch without APO. When compared with the patch containing colistin, the patch containing APO displayed accelerated wound healing and significantly reduced wound size [101]. Andreu *et al.* also investigated the nanofibers containing AMP. It can be appropriate for wound treatment in wound dressing [102]. Amariei *et al.* designed and prepared poly (acryl acid) and poly (vinyl alcohol) nanofiber containing e-polylysine as an AMP. They determined that the antimicrobial efficiency of these nanofibers with minimum inhibition concentration was in the following order: *S. epidermidis* > *S. aureus* > *E. coli* [103].

5.2.2.3 Polymer microgels, nanogels and hydrogels

Water *et al.* demonstrated that octenyl succinic anhydride-modified hyaluronic acid nanogels can apply as AMP (novicidin) in drug delivery systems. The maximum peptide loading of nanogels was 36±4%. The nanogels containing novicidin had reduced cytotoxicity, relatively high drug load, colloidal stability and showed the sustained release of drug over twelve days [104]. Silva *et al.* formulated hyaluronic acid nanogels containing AMP (LKKK18) and they demonstrated these nanogels can be applied in high therapeutic doses of the drug and display improved proteolytic stability [105].

Features such as high hydrophilicity, unique three-dimensional network, fine biocompatibility and cell adhesion make them suitable biomaterials for drug delivery in antimicrobial areas [106]. Various materials have been used in different researches for hydrogel preparation with AMP to target different organisms. Hydrogel formulations allow the sustained release of drugs, therefore, the incorporation of AMPs into these systems would offer prolonged AMP release at target sites and retain high AMP concentration in the nearby tissues [91]. In a research by Song *et al.,* an easily synthesized cell-adhesive hydrogel with inherent antibacterial activity was prepared as a potential scaffold for dermal wound healing based on chemical cross-linking between poly (Lys-Ala) polypeptides and 6-arm PEG-amide succinimidyl glutarate (ASG). As demonstrated in *Table II*, this hydrogel displayed significant antibacterial activity against *E. coli* and *S. aureus* [107]. Yang *et al.* prepared RADA-AMP (Tet213) self-assembling hydrogel, which can be appropriate for the treatment of bone infection and osteomyelitis [108].

5.3 Quality by Design based development

After the selection of the proper AMP, the extended Quality by Design (QbD) based development [109] is suggested (Figure 7). After the definition of the Quality Target Product Profile (QTTP), the next step is the initial risk assessment due to the complex interdependency of different factors and a number of possible risks [110, 111]. By means of proper quality management tools such as the Ishikawa diagram, the control of the Critical Quality Attributes (CQAs) and the Critical Process Parameters (CPPs) during modification and formulation of AMPs can be tested. The parameters can be divided into six groups: AMP characteristics, chemical modification method, final product characteristics, AMP formulation, therapeutic aim and administration rate, patient and AMP formulation. The application of Ishikawa diagram can high-

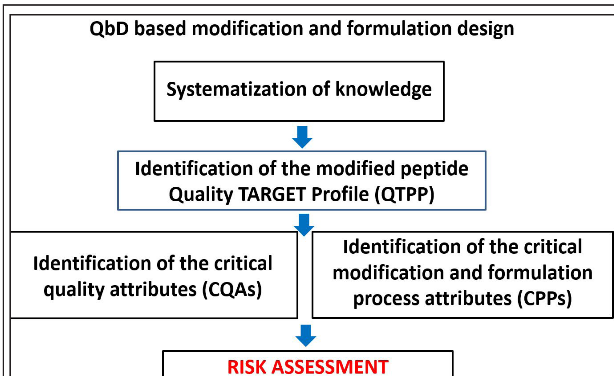

Figure 7 QbD based modification and formulation

light how identifying different factors that can affect the desired product quality, and thus the predefined AMP quality, including high metabolic stability, high cost efficiency, biocompatibility, low toxicity, low immunogenicity, retained antimicrobial activity and feasibility in reaching target sites at active concentration, is achievable.

6 Conclusions

As can be seen in *Table II*, it can be concluded that among different carrier systems, lipid-based nanocarriers offered a number of potential advantages as a delivery system for dermal or parenteral administration. The advantages include improved bioavailability of poorly soluble AMPs, enhanced hemocompatibility, high antimicrobial activity, high biodegradability and high selectivity of the peptide towards bacterial cells, protecting the cargo against unwanted interactions with the environment and providing a synergic effect. Besides this, these delivery systems have drawbacks, such as generation of undesired side products and limitations of controlling and triggering self-assemblies in lipid structure systems. On the other hand, recent scientific studies showed that polymer-based nanocarriers have several promising advantages, including enhanced antimicrobial activities of AMPs, low toxicity, low costs and selectivity of peptides for the target. Polymeric nanocarriers also showed disadvantages, such as possibility of inefficient delivery of AMPs to the target infectious site due to degradation. However, among different polymeric nanocarriers, hydrogels possess inherent antimicrobial activity and offer ease of synthesis and great potential to avoid secondary infections. Therefore it seems that among the above-mentioned delivery systems, lipid-based nanocarriers and polymeric hydrogels not only offer ease of synthesis, but their various advantages also outweigh their limitations and make them preferred nanocarriers in dermal and parenteral delivery systems of AMPs.

In this work, collecting and evaluating the results of various published researches led to achieving specifications in AMP delivery development. The knowledge of the physicochemical and structural features of AMPs facilitates the selection of a peptide with optimal features, such as: α-helical structure, amino acid charge between 3 to 6 and moderate amphipathicity and hydrophobicity, resulting in low toxicity and high antimicrobial activity.

Overall, in this review article different factors and possible associated risks in selection, modification and formulation of AMPs in a suitable delivery system were highlighted.

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Conflict of interest

The authors declare that there are no conflicts of interest.

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